DNA Strand Breaks in Human Nasal Respiratory Epithelium Are Induced upon Exposure to Urban Pollution

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All organisms have the ability to respond and adapt to a myriad of environmental insults. The human respiratory epithelium, when exposed to oxidant gases in photochemical smog, is at risk of DNA damage and requires efficient cellular adaptative responses to resist the environmentally induced cell damage. Ozone and its reaction products induce *in vitro* and *in vivo* DNA single strand breaks (SSBs) in respiratory epithelial cells and alveolar macrophages. To determine if exposure to a polluted atmosphere with ozone as the main criteria pollutant induces SSBs in nasal epithelium, we studied 139 volunteers, including a control population of 19 children and 13 adult males who lived in a low-polluted Pacific port, 69 males and 16 children who were permanent residents of Southwest Metropolitan Mexico City (SWMMC), and 22 young males newly arrived to SWMMC and followed for 12 weeks. Respiratory symptoms, nasal cytology and histopathology, cell viabilities, and single-cell gel electrophoresis were investigated. Atmospheric pollutant data were obtained from a fixed-site monitoring station. SWMMC volunteers spent >7 hr/day outdoors and all had upper respiratory symptoms. A significant difference in the numbers of DNA-damaged nasal cells was observed between control and chronically exposed subjects, both in children ($p<0.00001$) and in adults ($p<0.01$). SSBs in newly arrived subjects quickly increased upon arrival to the city, from $39.8 \pm 8.34\%$ in the first week to 67.29 ± 2.35 by week 2. Thereafter, the number of cells with SSBs remained stable in spite of the continuous increase in cumulative ozone, suggesting a threshold for cumulative DNA nasal damage. Exposure to a polluted urban atmosphere induces SSBs in human nasal respiratory epithelium, and nasal SSBs could serve as a biomarker of ozone exposure. Further, because DNA strand breaks are a threat to cell viability and genome integrity and appear to be a critical lesion responsible for p53 induction, nasal SSBs should be evaluated in ozone-exposed individuals. *Key words*: DNA damage, DNA single strand breaks, human nasal epithelium, ozone, urban pollution. *Environ Health Perspect* 104:160–168 (1996)

All organisms have the ability to respond and adapt to a myriad of environmental insults. Xenobiotics, chemicals, active oxidizing species, nonionizing and ionizing radiation, etc., are widespread in nature, and the whole spectrum of living forms have developed highly versatile protective systems to assure survival and reproduction (*1*).

The human respiratory epithelium, when exposed to oxidant gases in photochemical smog, is potentially at risk of DNA damage and requires efficient cellular adaptative responses to resist the environmentally induced cell damage. One example of such damage is the induction of DNA strand breaks, which represent a threat to cell viability, cell genome integrity, and may predispose to genomic alterations associated with neoplastic transformation and progression (*2*). Reactive oxygen species such as hydrogen peroxide (H_2O_2) have been shown to induce DNA strand breaks in a dose-dependent manner in a human bronchial epithelial cell line (BEAS-2B, S6 subclone) and in primary bronchial epithelial cells of nonhuman primates (*3,4*). Ozone (O_3) exposure *in vivo* of Fisher 344 rats induces single strand breaks (SSBs) in

alveolar macrophages (*5*), and a similar SSB induction is obtained when bronchioalveolar macrophages and tracheal epithelial cells of guinea pigs are exposed to 1.0 ppm O_3 for 2 hr (*6*). Hanley et al. (*5,7*) have suggested that ozone-degraded arachidonic acid (O_3-AA)-induced SSBs are primarily mediated by H_2O_2 and that nonaldehydic, non-hydrogen peroxide substances potentiate the H_2O_2-induced SSB effect. It has been shown that upon reaction with DNA, oxygen radicals produce more than 30 different adducts (*8*) and produce hundreds of different types of chemical changes in DNA that could give rise to mutagenic lesions (*9*) in a wide variety of cell systems (*10–14*).

We have studied the human nasal respiratory epithelium chronically exposed to high ozone concentrations to determine the adaptative responses of this type of epithelium to environmental insults and, more importantly, to determine if these responses are active participants in a sequence of events that could eventually lead to overt malignancy (*1*). Of particular interest to us has been the work of Lee and Hanley et al. (*4–7*) concerning the *in vivo* and *in vitro* correlation of ozone exposure

and the induction of SSBs in respiratory cells, both in experimental animals and in humans. Recently, a report by Nelson and Kastan (*2*), indicating that DNA strand breaks appear to be a critical DNA lesion responsible for p53 induction in cells with wild-type p53 alleles exposed to DNA-damaging agents, has opened several research avenues for the study of pollutant-exposed human respiratory epithelia.

The aims of this study were 1) to investigate if children and young adults chronically exposed to a polluted urban atmosphere (Southwest Metropolitan Mexico City; SWMMC) with ozone as the main criteria pollutant have more DNA SSBs in their nasal respiratory epithelia, compared to control populations living in a low-polluted environment and 2) to study the kinetics of nasal cells SSBs induction and the nasal histopathology of a population of young, healthy males newly arrived to SWMMC and followed for 12 weeks. In this context, we also explored if there was a correlation between cumulative ozone concentrations and nasal SSB induction.

Methods

Study population. This project was approved by the Instituto Nacional de Pediatria (INP) Review Boards for Human Studies, and informed written consent was obtained from the subjects or their parents. The study group consisted of 139 volunteers, including a control population of 13

adult males and 19 children (9 females, 10 males) who lived in Manzanillo, a low-polluted Pacific port, 69 young adult males and 16 children (8 females, 8 males) who were permanent residents of Southwest Metropolitan Mexico City (SWMMC) and 22 young adult males newly arrived to SWMMC and followed for 12 weeks from their time of arrival.

All participants in the study were healthy nonsmokers, with a no history of smoking or only passive exposure to tobacco at work or at home. Control subjects were permanent residents in a small, low-polluted port city; these children and adults seldom left their small town, had never been to a large city (including Mexico City), and had no known exposures to local sources of air pollutants or toxic substances.

All volunteers and children's parents were interviewed by two researchers, and information was obtained about age, sex, place and length of residency, socioeconomic status, daily outdoor time, level of physical activity and time of the day when it took place, dietary intake, alcohol consumption, household cooking methods, occupational history, history of toxic exposures, cigarrette smoking, family household smoking habits, family history of respiratory disease, history of allergy, respiratory and otolaryngological symptoms, including epistaxis, rhinorrhea, quantity and quality of nasal mucus, nasal dryness, nasal obstruction, cough, thoracic pain, difficulty breathing, and recent acute respiratory illness (in the previous 3 months).

Children and adults with a history of ear-nose-throat surgical procedures or in need of treatment, atopic or infectious rhinitis, bronchitis, asthma, allergic diseases, known household or work exposures to potentially harmful substances (i.e., solvents, paints, metals, photocopying machines), frequent travelers outside their residential area, those taking medications (including vitamins), and overweight subjects were excluded from the study population. Information on socioeconomic status included the adult subject's usual occupation and his educational level. Information on diet and alcohol consumption was gathered by means of a semiquantitative food-frequency questionnaire and available diet records. Four categories of alcohol intake were defined: less than one drink per month, one or more drinks per month but less than one per day, one or more drinks per day but less than three per day, and three or more drinks per day. There were no subjects in the latter category.

Control populations. The control population included 19 children, age 11.21 ±

0.52 years with a daylight outdoor exposure of 6.26 ± 0.98 hr and 13 adult males, age 24.46 ± 2.6 years with a daylight outdoor exposure of 11.2 ± 1.4 hr.

SWMMC study subjects. Southwest Metropolitan Mexico City adult residents were divided into four groups according to their place of enrollment: group 1: (n = 43) enrolled at a Security Corporation, consisted of healthy males, age 24.46 ± 3.41 years (range 18–36), with a daylight outdoor exposure of 11.6 ±1.2 hr and an SWMMC average residency time of 116 months; group 2: (n = 12) enrolled at the Instituto Nacional de Pediatria, age 32 ± 10.15 years (range 17–47), daylight outdoor exposure of 10.9 ± 1.4 hr and an SWMMC average residency time of 210 months; group 3: (n = 14) enrolled at a soft drink distributer, age 26.57 ± 7.53 years (range 18–42), daylight outdoor exposure 11.2 ± 1.8 hr and an SWMMC average residency time of 231 months; group 4: (n = 22), age 23.18 ± 2.6 years (range 21–29) and daylight outdoor exposure of 11.6 ± 1.2 hr. Group 4 volunteers were enrolled at the same Security Corporation as group 1 and were residents in small towns across the country; they arrived at SWMMC the same day and were followed for a 12-week period. These subjects had never been to Mexico City or any major city, and the criteria for their inclusion in the study were the same as for the other groups. They were immediately housed in well-ventilated buildings that used open windows for cooling and without local sources of toxic substances. The living-working housing and the premises were enclosed, so vehicular traffic was minimal.

The adult control group and groups 1 and 4 had an identical dietary intake, level of physical activity, and exercise routine outdoors. These subjects stayed outdoors from 0600 to 1800 hr, with only brief intermittent periods indoors (less than 1 hr total). Six days per week, from 0800 to 1400 hr, they exercised moderately outdoors and had intermittent, light exercise the remaining of the outdoor time.

SWMMC children. SWMMC children (n =16), age 12.18 ± 0.63 years had an outdoor exposure time of 7.75 ± 1.3 hr. These children were lifelong residents in SWMMC, and all attended the same school and lived in the same neighborhood.

None of the volunteers showed evidence in their medical histories or after a complete physical examination of any viral illness or respiratory disease. Group 4 subjects were in good health throughout the 12-week study period.

Nasal biopsies. We obtained samples of

nasal respiratory epithelium with a disposable plastic curette (Rhino-probe, ASI, Arlington, Texas) under direct visual inspection. With gentle pressure, the probe was drawn along the inferior surface of the inferior nasal turbinate several times and retracted. The nasal epithelium obtained by this procedure was in the form of oval clusters of well-oriented cells, yielding up to 1 million cells as determined by an hemocytometer. The procedure proved to be simple and minimally invasive, producing little discomfort, occasional tearing of the ipsilateral eye, and sneezing after the procedure. Two nasal biopsy samples were obtained from individuals in control groups and exposed groups 1–3, while for subjects in group 4 we took two biopsies at each sampling time (0, 1, 2, 4, 8, and 12 weeks after arrival in SWMMC). One sample was immediately immersed in 1 ml of cold RPMI 1640 medium (Gibco, Grand Island, New York) and reserved for the viability assay, cell count in the hematocytometer, single-cell electrophoresis assay, flow cytometry, and fresh observations of ciliary motility. The second biopsy sample taken from the opposite side was divided in two: one portion was fixed in 10% formaldehyde for light microscopic studies, and the other was fixed in glutaraldehyde for electron microscopy further studies. SWMMC nasal samples were easily disaggregated by gently shaking the glass tube; control samples and the first sample in group 4 required mincing with a scalpel blade and vortexing for 10 sec. Using a hematocytometer, 10 μl of the cell suspension was taken to perform a cell count and a 100 μl was used for a viability test with the propidium iodide exclusion assay in an EPICS, Profile II Coulter flow cytometer (Coulter, Hialeah, Florida) (*15*). Observations were made as to the overall adequacy of the single-cell separation, distribution of cell types, and ciliary motility.

Single-cell gel electrophoresis assay. We assessed DNA SSBs by the single-cell electrophoresis (SCGE) assay (*16,17*). Briefly, the single nasal cell suspension volume was adjusted to 50,000 cells/50 μl. These cells were mixed with 50 μl of low melting point agarose at 37°C and then placed on precleaned microscope slides (Fisher fully-frosted slides, Fisher Scientific, Pittsburgh, Pennsylvania) which were already covered with a thin layer of 0.5% normal-melting agarose. The slides were kept at 4°C for 5 min to allow solidification of the agarose and then immersed in a freshly made cold-lysing solution (1% sodium sarcosinate, 2.5 M, NaCl, 100 mM, Na$_2$-EDTA, 10 mM Tris, pH 10, and 1% Triton X-100) for 1 hr to lyse the cells and to permit DNA

unwinding. The slides were then removed from the lysing solution and placed on a horizontal gel electrophoresis unit (Easy Cast, Model B2, Owl Scientific, Inc., Woodburn, Massachusetts). The unit was filled with fresh electrophoretic buffer (1 mM EDTA and 300 mM NaOH), and the slides were allowed to set for 20 min to permit unwinding of DNA before electrophoresis. Electrophoresis was conducted for the next 20 min at 25 V using an electrophoresis compact power supply (Buchler Instruments, Kansas City, Missouri). All the steps described above were conducted under yellow light. After electrophoresis the slides were washed gently with 0.4 M Tris, pH 7.5, and then stained each with 25 µl of 20 µg/ml ethidium bromide in distilled water. Slides were viewed within 2 hr of this last step. Observations were made using an Olympus AH-2 microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. We analyzed a minimum of 50 randomly selected cells per sample and the score was calculated manually through the use of an ocular micrometer by two independent observers. To quantitate DNA migration, we measured the tail length of individual cells in two different slides from the same nasal biopsy sample. The length of migration included the size of the nucleus; to elaborate the histograms of the distribution of the migration length, we arbitrarily divided the scale in four groups: 10–40 µm, 41–80 µm, 81–120 µm and >120 µm.

Light microscopy. Samples were processed in paraffin, cut at 5 µm and stained with hematoxylin and eosin (H&E). Cell suspensions used for the SCGE assay were stained with Papanicolau stain.

Pollutant methodology. Atmospheric pollutants and meteorological conditions were monitored at the Pedregal station located in SWMMC, downwind of the major diurnal emissions in Metropolitan Mexico City and 3 miles or less from the SWMMC volunteers' residence, school, and workplaces. Ozone was monitored using a Beckman 950 chemiluminescence analyzer with a calibration routine in accordance with U.S. EPA procedures. We added the hourly average values, equal or greater than 0.06 ppm (SUM_{06}) ozone from 1 March to 31 May 1995 for the outdoor exposure time (0600–1800 hr) applicable to exposed group 4. We also monitored NO_2, NO_x, SO_2, CO, temperature, relative humidity, wind speed, and rain events. The maximal concentrations, number of hours equal or above the National Ambient Air Quality Standards (NAAQS), and the time of ocurrence of pollutant peaks were recorded. Data from

Manzanillo were obtained from the Capitania del Puerto.

Statistical analysis. Data were analyzed in three ways: nonparametric Kolmogarov-Smirnov test to compare the accumulated frequencies of SSBs in control versus exposed subjects; nonparametric Mann-Whitney U-test to compare the percentage of DNA-damaged cells in control versus exposed children; and analysis of variance to compare the percentage of cells with DNA damage in control versus the different exposed adult groups, followed by Dunnett's test. Results are given as means ± standard deviations. A p-value of <0.05 was used to determine statistical significance.

Results

Air Quality Data

Permanent residents of SWMMC have been recurrently exposed to ozone, the main criteria pollutant in the area. In the last 8 years, ozone concentrations at and above the current NAAQS for ozone (0.12 ppm as 1 hr maximum concentration, not to be exceeded more than once per year) have been recorded in SWMMC every day, an average of 3 ± 1.0 hr/day (*18*). The number of hours SWMMC subjects are exposed to ozone above the NAAQS has been 740, 959, 1224, 1403, 1561, 1395, 1146, and 1061 hours, for the years 1987–1994. SWMMC volunteers were studied in a 6-month period (November 1994–May 1995) characterized by an average of 4.40 hr per day of ozone > 0.12 ppm.

The maximum ozone concentration recorded in this period was 0.305 ppm (December 1994), and the average maximum concentration for the period was 0.269 ppm. NO_2 and sulfur dioxide (SO_2) concentrations for the study period were usually below 0.053 and 0.03 ppm, respectively. Temperature and relative humidity (RH) were within the normal range for the seasons (16–26°C and RH of 45–65%). For group 4 volunteers, we calculated the SUM_{06} for ozone (all hourly average values equal to or greater than 0.06 ppm) for the daily exposure period 0600–1800 hr, 1 March through 31 May 1995. Table 1 shows the relevant data for this period; the average maximum ozone concentration was 0.262 ppm, subjects were exposed to 413 hr of ozone above 0.12 ppm, and the SUM_{06} was 88.42 ppm-hr. Values at or above 0.06 ppm O_3 were recorded between 0900 and 1000 hr and persisted up to the time the volunteers returned to their quarters (1800 hr).

Sulfur dioxide concentrations were usually below 0.03 ppm, the higher values recorded were 0.076, 0.093, and 0.054 for

the months of March, April, and May, respectively. NO_2 concentrations < 0.053 ppm were usually recorded. The control population in the Pacific port was sampled in January 1995 with atmospheric and metereological conditions average for the season: 26°C, RH 87%, wind speed 9–18 km/hr and no detectable atmospheric pollutants.

Study Populations: Clinical Data

Nasal and respiratory symptomatology were absent in the control groups. Table 2 shows the clinical features for control and exposed subjects. Nasal atrophy in children was a prominent finding, while in adults, group 3 showed the highest atrophy frequency. Epistaxis was present in variable degrees in all SWMMC subjects, but was particularly prominent in group 1. Nasal obstruction and nasal dryness were similar in all exposed groups; cough and thoracic pain were frequent complaints in children and were associated with exercise outdoors. Intermittent increased nasal mucus was a universal complaint in all exposed volunteers.

Group 4 subjects experienced nasal symptoms as early as 24 hr after arrival in SWMMC; epistaxis was an important complaint for the first 2 weeks of residency. Nasal mucus and nasal dryness along with thoracic pain were the most common symptoms after week 2 and up to the last study week.

For control adults and SWMMC group 1 subjects, we had access to the records of their daily diets for the previous 3 years. Subjects in group 4 had access to the same diet from the day they arrived in Mexico City. We computed the mean daily intake for 4 random periods of 1 week during the previous year as the primary unit of measurement. As seen in Table 2, there is a significant difference between the caloric intake in controls and exposed groups 1 and 4 versus subjects in groups 2 and 3. Controls and groups 1 and 4 volunteers had access to 14 servings of citrus fruits, 8 servings of seasonal fruits (excluding juice), and 22 servings of vegetables per week, whereas group 2 subjects had 4.2, 4.5, and 7.8 and group 3 subjects that had 2.2, 2,

Table 1. Atmospheric exposure data for group 4, newly arrived volunteers to Southwest Metropolitan Mexico City

Study month	$O_3 > 0.12$ ppm Per month (hr)	Per day (hr)	Maximum O_3, ppm	$O_3 SUM_{06}$,[a] (ppm-hr) 0600–1800 hr
March	134	4.32	0.266	26.976
April	104	3.46	0.272	26.025
May	175	5.83	0.250	35.419

[a]Cumulative O_3 values ≥0.06 ppm-hr.

Table 2. Clinical characteristics of control and exposed subjects

Characteristic	Children Control (n = 19)	Children Exposed (n = 16)	Control adults (n = 13)	Group 1 (n = 43)	Group 2 (n = 12)	Group 3 (n = 14)
Age (years)	11.2 ± 0.52	12.18 ± 0.63	24.46 ± 2.6	24.5 ± 3.41	32 ± 10.15	26.57 ± 7.53
Male (%)	52.63	50	100	100	100	100
Outdoor exposure (hr)	6.36 ± 0.98	7.75 ± 1.72	11.2 ± 2.1	11.6 ± 1.2	10.9 ± 2.5	11.2 ± 1.8
Residency SWMMC (months)	0	146	0	116	210	231
% Nasal cells with SSBs	17.09 ± 6.07	63.55 ± 13.37	14.74 ± 7.96	67.57 ± 6.25	91.56 ± 9.13	90.27 ± 6.22
Nasal mucosa atrophy (%)	0	75	0	9	25	36
Epistaxis (%)	0	25	0	56	25	43
Nasal mucous (%)	0	100	0	100	100	100
Nasal obstruction (%)	0	50	0	58	67	57
Nasal dryness (%)	0	44	0	42	66	43
Cough (%)	0	87	0	67	75	50
Thoracic pain (%)	0	50	0	9	25	29
Total calories/day	2042	1835	2680	2765	1880	1137
Alcohol intake (I–IV)[a]	0	0	10 (0) 2 (I) 1 (II)	36 (0) 2 (II) 5 (III)	9 (0) 1 (I) 2 (II) 3(III)	8 (0) 1 (I) 2 (III)

Abbreviations: SWMMC, Southwest Metropolitan Mexico City; SSBs, single-strand breaks.
[a]Categories of alcohol intake: I, less than one drink per month; II, one or more drinks per month, but less than one per day; III, one or more drinks per day, but less than three per day, and IV, three or more drinks per day.

Table 3. DNA nasal cell damage in control versus exposed subjects

Study populations	% Nasal cells with SSBs
Control adults	14.75 ± 2.06
Control children	17.09 ± 6.07
SWMMC children	63 ±13
SWMMC group 1	68.28 ± 5.94
SWMMC group 2	91.56 ± 9.13
SWMMC group 3	90.27 ± 6.22

Abbreviations: SWMMC, Southwest Metropolitan Mexico City; SSBs, single-strand breaks.

Figure 1. Adult control and exposed volunteers (groups 1–3) and proportions of normal and DNA-damaged cells (length of single-strand breaks). Note that subjects in groups 2 and 3 have the most DNA damage with similar extended DNA migration patterns.

and 3.4 servings per week, of citrus fruits, fruits, and vegetables, respectively. There were also differences between control and exposed children in their servings of citrus fruits (12.6 versus 6.2), fruits (5.2 versus 2.4), and vegetables (12.6 versus 4.8) per week. It is important to note that adult volunteers in the control group and exposed groups 1 and 4 had stable, normal weights.

Single Cell Gel Electrophoresis

A significant difference ($p<0.00001$) in the numbers of DNA-damaged cells was observed between control and exposed children (Table 3). The number of nasal cells with SSBs in control children was small 17.09 ± 6.07%, and all of them migrated in the range of 10–40 μm (no different from the results in control adults, $p=$ 0.3854). In contrast, SWMMC children showed 63 ± 13% cells with SSBs, migrating mostly in the range of 41–120 μm.

Similar results were obtained with the adult populations (Table 3), with significant differences between control and exposed subjects (groups 1–3 $p < 0.01$; Fig. 1). Interestingly, groups 2 and 3 had similar
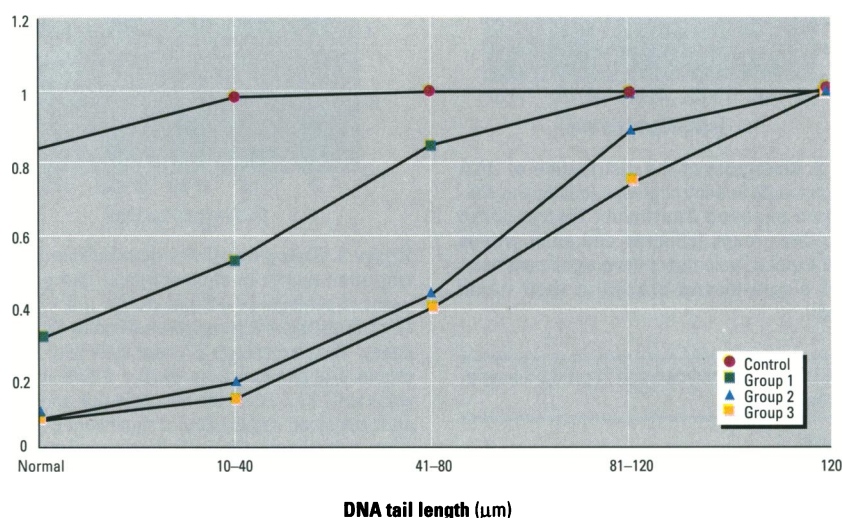
numbers of DNA-damaged cells ($p =$ 0.5968), but there was a statistical difference between group 1 and groups 2 and 3 ($p< 0.01$). Figure 2 shows the histogram of the distribution of DNA migration length in control and exposed children and adults. Adults in groups 2 and 3 had the most DNA damage, with the majority of their nasal cells exhibiting extended DNA migration patterns. Exposed children and the adults in groups 1 and 4 (week 12) showed a similar pattern of DNA migration.

Group 4 results. There was a significant increment in the number of SSBs in the nasal cells of these volunteers at weeks 1 and 2 of their arrival in SWMMC, thereafter the percentage of damaged cells

remained stable (Table 4). Interestingly, SUM_{06} ozone for week 1 was 3.716 ppm·hr, for week 2, the cumulative value was 9.697 ppm·hr, and by week 12 this value had reached 88.42 ppm·hr. Figure 3 shows the DNA migration patterns for weeks 1–12. We did not observe any significant differences in the patterns between weeks 4 and 12.

Cell Viability, Cytological Observations, and Histopathology

Control adults and children had similar nasal cell viabilities as evaluated by the propidium iodide exclussion assay: 72.5 ± 6.3% for children and 76.2 ± 8.2% for adults. Exposed children and adults in
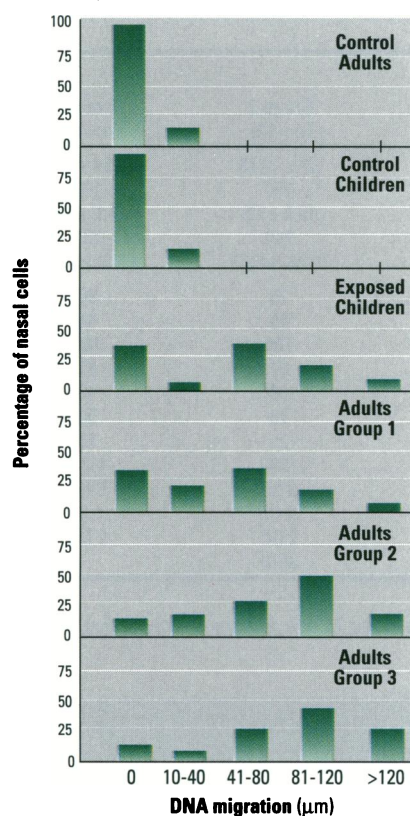
Figure 2. Histogram of the distribution of DNA migration length in control groups (adults and children) and exposed Southwest Metropolitan Mexico City groups (children and adult groups 1–3). All exposed volunteers have significant numbers of single-strand breaks in their nasal epithelia.

Table 4. DNA nasal cell damage in group 4, newly arrived subjects

Residency time in SWMMC (weeks)	% SSBs	Cumulative SUM$_{06}$[a] (ppm-hr)
1	39.80 ± 8.34	3.716
2	67.29 ± 2.35	9.697
4	63.36 ± 2.78	26.976
8	65.67 ± 5.25	53.01
12	62.42 ± 3.34	88.42

[a]Hourly average concentrations ≥ 0.06 ppm O_3 for the volunteers outdoor daily exposure time (0600–1800 hr).

groups 1–3 had lower cell viabilities: 62.8 ± 10.98. For group 4 subjects, the lowest cell viability was recorded at week 1: 60.3 ± 9.2, while from week 2 through 12 the cell viabilities were on average 65.2 ± 8.2. Nasal specimens placed in RPMI medium were evaluated by inverted light microscopy with special emphasis on ciliary motility. Control subjects had abundant cilia with strong, regular beating movements, whereas exposed subjects had only a few short cilia left, with weak and irregular
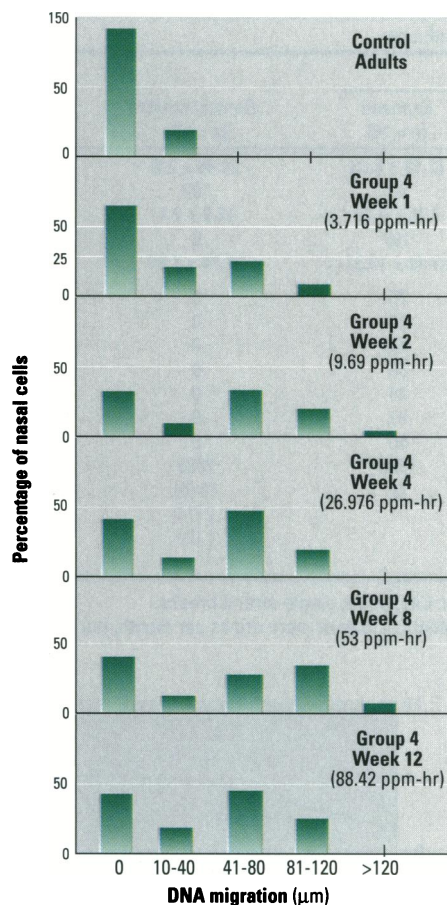

Figure 3. Histogram of the distribution of DNA migration length in control adults and group 4 exposed subjects (n = 22) at weeks 1, 2, 4, 8, and 12 of Southwest Metropolitan Mexico City residency. The percentage of nasal cells with single-strand breaks went from 39.80 ± 8.34% the first week to 67.29 ± 2.35% the second week and thereafter remained stable without significant changes. Data based on >50 cells/per nasal sample. Cumulative O_3 values ≥0.06 ppm-hr in parentheses.

beating. A cobblestone appearance was a common finding in the exposed samples; luminal cells had a cuboidal appearance with no visible cilia by light microscopy (Fig. 4A). Some epithelial cells showed multiple blebs on their cytoplasmic surfaces; this observation was particulary prominent in the fresh nasal samples from exposed children.

An average of 2 mm of nasal epithelium was present in the 139 biopsies examined. In control subjects, normal mucociliary epithelium was present (Fig. 4B); exposed children for the most part had cohesive samples, where respiratory ciliary epithelium could still be identified (Fig. 4C). Polymorphonuclear cells could be seen infiltrating the hyperplastic epithelium; in addition, the majority of the children's samples showed scattered single cells with compaction and segregation of the nuclear

chromatin, intranuclear, sharply delinated, uniformly granular masses marginated against the nuclear envelope, and condensation of the cytoplasm (Fig. 4C,D). Adult exposed biopsies showed, in addition, luminal necrotic cells characterized by a swollen cytoplasm and disintegrating plasma and organelle membranes; and squamous metaplastic epithelium completely substituted for the normal mucociliary cells (Fig. 4E).

The sequential biopsies in the newly arrived subjects showed interesting changes. By week 1, there was already a complete loss of cilia, and the epithelial cells displayed large nucleoli and a finely vacuolated cytoplasm (Fig. 5A). By week 2, a cobblestone appearance was evident, with apical accumulation of secretory granules (Fig. 5B). An interesting finding at week 2 was the presence of small blood vessels within the epithelium (Fig. 5C), the apparent result of neovascular proliferation, at a time when the subjects complained of epistaxis. Also at week 2, intercellular bridges were obvious, and there was a marked decrease in the number of goblet cells. Samples from weeks 4, 8, and 12 showed squamous metaplastic changes, mild to moderate nuclear pleomorphism, and large and often multiple nucleoli (Fig. 5D).

Discussion

The results of this study demostrate that exposure to a polluted urban atmosphere with high ozone concentrations induce the formation of DNA SSBs in the nasal respiratory epithelium of children and adults. Long-time SWMMC residents have a high proportion of nasal cells with SSBs, and SSBs in newly arrived young adults quickly increase during the first 2 weeks in the polluted environment. Oxidative stress from air pollution is an important mechanism of toxicity, if not the only one, in the human respiratory tract (19). Ozone is a strong oxidizing agent that interacts with a wide variety of organic molecules to produce toxic free radical intermediates, initiate cascades of free radical reactions that damage genetic integrity, and at relatively high concentrations cause extensive DNA damage as reflected by strand breaks, DNA interstrand-crosslinks, and DNA–protein crosslinks (9,10,20–22).

Hanley et al. (5) reported that O_3-AA induced DNA SSBs in cultured human lung fibroblasts, the primary causative agent being hydrogen peroxide, and suggested that the nonaldehydic portion of O_3-AA is responsible for potentiation of SSBs (7). Human bronchial epithelial cells (BEAS, subclone R1-4) and nonhuman primate bronchial epithelial cells incubated

in H_2O_2 showed dose-dependent increases in strand break formation (*3*). DNA strand breaks are caused by cleavage of phospho-diester linkages in one of the polynucleotide chains; destruction of the deoxyribose ring can also result in ruptures of the deoxyribose-phosphate backbone. The presence of a DNA SSB causes localized denaturation and increases the probability of free radical attack at this site due to the loss of the protective effect of base-stacking interactions (*23*).

Oxidants can produce direct DNA strand breaks and DNA damage that is processed into DNA strand breaks by cellular repair enzymes (*24*). DNA strand breaks activate the chromosomal enzyme poly(ADP-ribose)polymerase to use NAD for the production of poly(ADP-ribose), which in turn facilitates the DNA repair process. At low levels of DNA damage, synthesis of the polymer facilitates the DNA repair process, but when the DNA damage is severe and persists for long periods, there is consumption of the substrate NAD and drastic alterations in cellular metabolism, and ultimately the cells may undergo swelling and degeneration within several hours of sustaining DNA damage (*25*).

The persistence of DNA damage may transmit signals to other cellular components including p53 (*11*). Agents that rapidly induce DNA strand breaks rapidly trigger p53 protein elevations. Nelson and Kastan (*2*) reported that DNA strand breaks are sufficient and probably necessary for p53 induction in cells with wild-type p53 alleles exposed to DNA-damaging agents. This finding is extremely relevant to oxidant-induced damage to the nasal mucosa because p53 wild-type protein accumulates to high levels within nasal squamous metaplastic cells of SWMMC-exposed subjects (LC-G, personal observation).

Major functions of wild-type p53 protein include the regulation of a G_1-S cell cycle check-point and the mediation of apoptosis (*26–30*). Thus, the presence of DNA strand breaks in nasal cells could
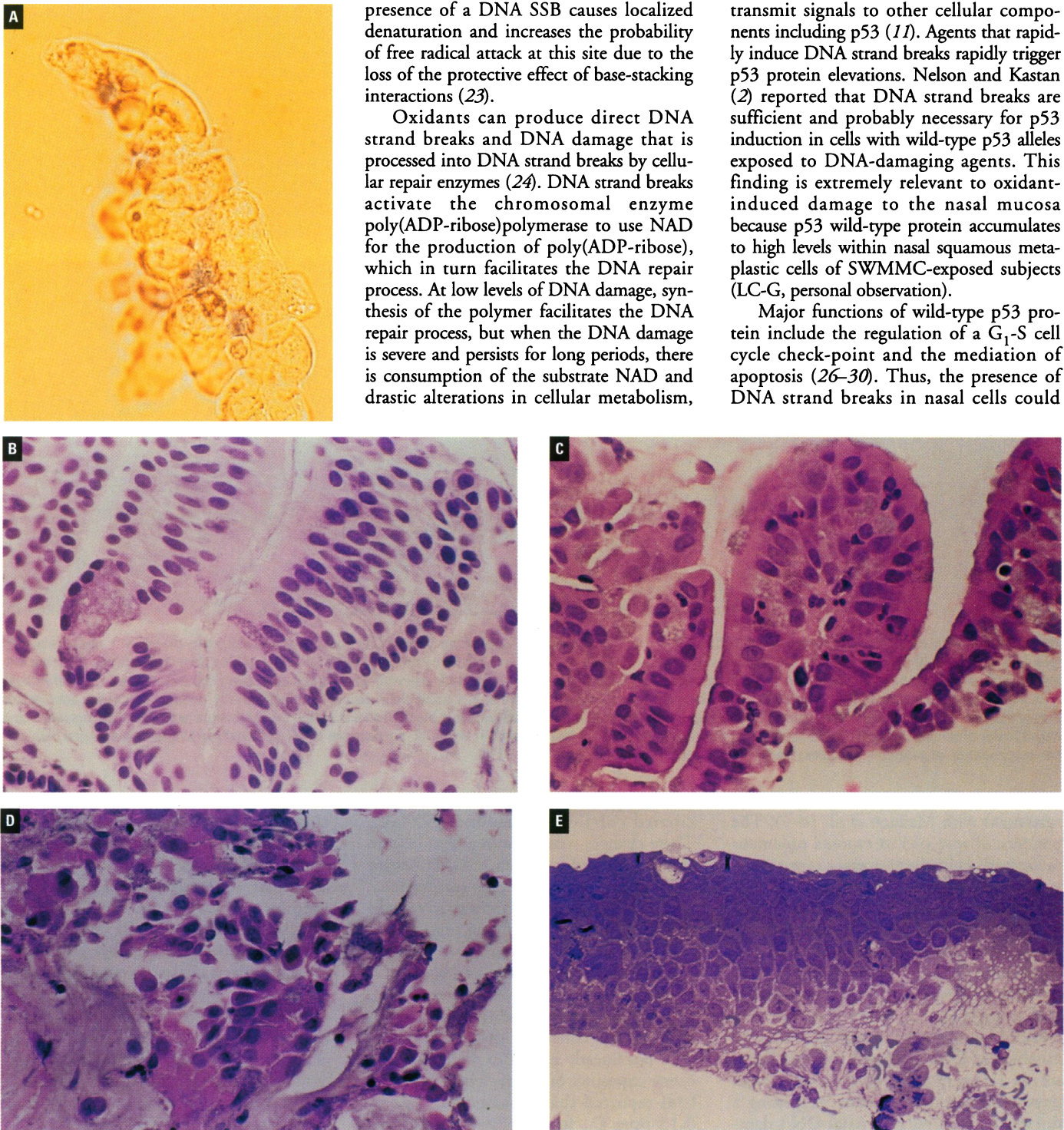
Figure 4. Cytology and histopathology of nasal epithelial samples. (A) Cluster of fresh nasal epithelial cells stained with trypan blue; a cobblestone appearance is seen on the luminal surface, where there are no visible cilia (H&E, ×90). (B) Control nasal biopsy. Note the luminal surface lined by normal, intact ciliated and goblet cells (H&E, ×114). (C) Nasal biopsy from an exposed child; the cells on the luminal surface are flat and a few short cilia are seen. Polymorphonuclear cells infiltrate the epithelium, and scattered cells with compacted nuclear chromatin and cytoplasmic condensation are present (H&E, ×114). (D) Fragmented nasal sample from an exposed child; notice the deeply eosinophilic and granular cytoplasm and the mild variation in nuclear size (H&E, ×116). (E) Nasal biopsy from an exposed adult (group 3). Notice that squamous metaplastic epithelium has completely replaced the normal respiratory ciliated-type epithelium. The luminal surface has a cobblestone appearance with no cilia and a few luminal cells display a clear cytoplasm and disintegration of organelles and plasma membranes. There is moderate variation in nuclear size with prominent nucleoli. A cluster of macrophages with dark purple intracytoplasmic material is also seen. (H&E, ×91).
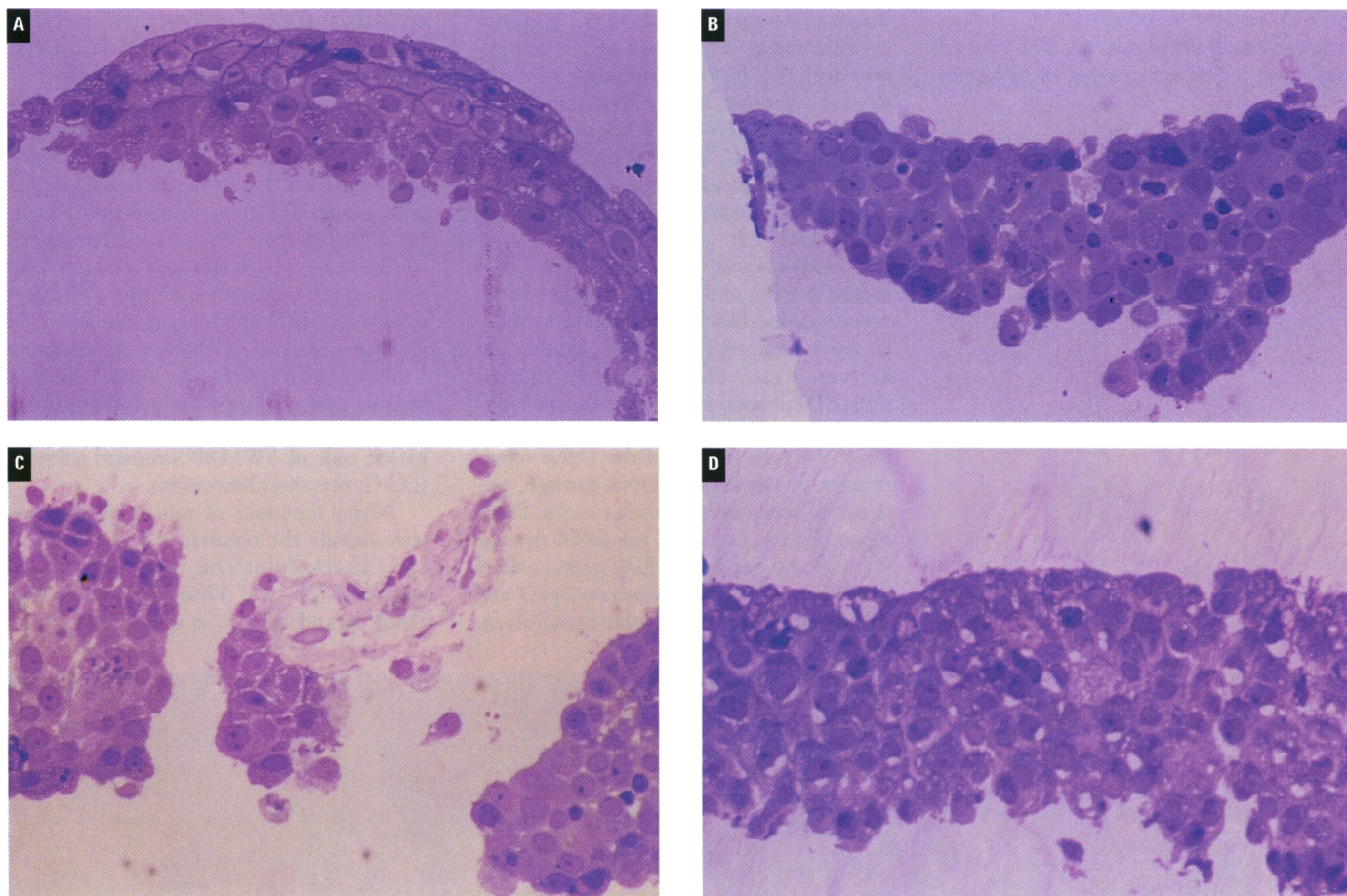
Figure 5. Sequential nasal biopsies from a 22-year-old male arriving to Southwest Metropolitan Mexico City from a low-polluted town. (A) Week 1: nasal cells with total loss of cilia, cobblestone appearance, nuclei with large nucleoli and a finely vacuolated cytoplasm. (B) Week 2: the cobblestone appearance is prominent, luminal cells show apical accumulation of secretory granules. There is mild variation in nuclear size with large nuclei containing multiple nucleoli. Scattered cells with condensed cytoplasm and compacted nuclear chromatin are also seen. (C) Week 2: a small blood vessel with hyperplastic endothelial cells is seen in this biopsy fragment. Epithelial cells with intercellular bridges are attached to the vessel wall; this blood vessel is within the epithelium proper and corresponds to a neovascular projection through the basement membrane, which was not trespassed. (D) Week 12: persistence of the cobblestone luminal appearance with apical accumulation of secretory granules; there is mild variation in nuclear size (A–D, ×114).

serve as a biomarker of ozone exposure in agreement with Madden et al. (4–7). The presence of wild p53 in exposed squamous metaplastic epithelium opens a new avenue worth exploring in pollutants research.

An interesting but not unexpected finding in this work was the initial linear correlation between an increase in SSBs and cumulative (SUM_{06}) ozone exposure (31). By week 3, however, when the normal ciliated epithelium with goblet cells had been replaced by squamous metaplastic cells, the numbers of DNA damaged cells reached a steady state, in spite of the continuous increase in cumulative ozone, suggesting a threshold for cumulative nasal DNA damage. A similar epithelial damage threshold to oxidant injury has been observed in experimental animals. Hotchkiss et al. (32) assessed the effect of different cumulative exposure times on ozone-induced rat nasal epithelial hyperplasia; three 6 hr/day exposures to 0.8 ppm O_3 triggered hyperplastic

and metaplastic changes in the nasal nonciliated cuboidal epithelium (NNCE). These changes were indistinguishable from those produced by seven 6 hr/day exposures to the same ozone concentrations. The authors suggest that sensitive cells within the NNCE were likely injured during the first 3 days of exposure to 0.8 ppm O_3 and that repair introduced a new O_3 resistance population. Henderson et al. (33) measured ozone-induced nasal DNA synthesis and concluded that the responses were not linearly related to the cumulative ozone exposure. Similarly, Harkema et al. (34) reported that monkeys exposed to 0.15 ppm O_3 for 6 or 90 days did not show any significant difference in epithelial response (hyperplasia of nonciliated cuboidal epithelial cells), suggesting a threshold in the bronchiolar epithelium.

A factor probably accounting for the DNA SSB steady state is the development of a squamous metaplastic epithelium, a

new cell population associated with resistance to environmentally induced damage. This metaplastic epithelium could correspond to what Farber and Rubin call a type B cellular adaptative response (1). This resistant new cell population is likely to have biochemical-molecular changes, such as a biochemical pattern that diminishes the toxic effects of xenobiotics. Changes in antioxidant enzyme patterns could be included as key factors in the resistance features. Plopper et al. (35) have described changes in glutathione transferase, glutathione peroxidase, and superoxide dismutase in the lungs of rats in response to long-term exposure to O_3; the changes varied by site within the airway tree, and there were significant age-related changes in activities of the antioxidant enzymes. Antioxidants located in the lining layer of the respiratory tract may also be important in determining resistance of epithelia to inhaled pollutants (36). An interesting observation in this

regard is the severe nasal damage present in the exposed adult group with a poor intake of vegetables and fruits as compared with a similarly exposed group with a balanced diet rich in these nutrients. Moreover, the newly arrived subjects to SWMMC were given the same balanced diet, and, although they had DNA damage, the damage did not reach the severity of the subjects eating a poor diet. Although the focus of this paper was not primarily to study the influence of a properly balanced diet upon air pollutant damage in the respiratory tract, it is obvious that a diet rich in selected vegetables and fruits has a protective effect, a subject that deserves further investigation in chronically ozone-exposed populations.

It is well established that angiogenesis is an important factor in normal wound healing and in the process of carcinogenesis (37,38). Induction of angiogenesis has been shown in the transition from hyperplasia to neoplasia in experimental animals (39). Proliferating capillaries were seen within the nasal epithelium of volunteers as early as 2 weeks after arrival in the city; previous work in our laboratory has shown proliferation of thin vascular submucosal channels as well as capillaries within the basement membrane or projecting toward the epithelium in SWMMC residents with <30 and >60 days in the city (40), and many of these subjects had recurrent episodes of epistaxis. Laniado-Schwartman et al. (41) demostrated that an arachidonic acid metabolite, 12(R)-hydroxyeicosatrienoic acid, is an angiogenic factor in microvessel endothelial cells. Dameron et al. (42) showed that the switch to the angiogenic phenotype by fibroblasts obtained from Li-Fraumeni patients coincided with the loss of the wild-type allele of the p53 gene and was the result of reduced expression of thrombospondin-1. It remains to be seen what role the different angiogenic factors play in the ozone-exposed respiratory epithelium of both experimental animals and in humans.

Another aspect of this study was the use of the sum of all hourly average O_3 concentrations > 0.06 ppm (SUM_{06}) as a measurement of ozone exposure, instead of only considering the number of hours at or above the NAAQS or the maximum peak concentrations. We believe that the use of a cumulative exposure index over an extended period of time is an appropiate approach to link cause-and-effect relationships between ozone and human health. In our population, exposed several hours a day to high ozone concentrations, the issue is probably not that crucial; however, in populations exposed fewer hours or to lower ozone concentrations, additional attention should be given to exposures that accumulate over time.

In closing, it should be emphasized that the human nasal respiratory epithelium responses to inhaled pollutants are very complex and studies like this one have obvious limitations because of the heterogeneous study population, the differences in nutritional intake and outdoor exposure patterns, and the lack of personal ozone passive samplers to estimate ozone exposures accurately. However, the results of this study do indicate that SSBs in human nasal respiratory epithelium are induced upon exposure to a polluted urban environment. The finding that DNA strand breaks are sufficient to trigger p53 induction and the suggestion that strand breaks predispose to genomic alterations associated with neoplastic transformation and progression (2,26–30,43) suggests that we should identify in these highly ozone-exposed populations the presence of cell-cycle alterations and/or apoptotic cell death pathways, as well as the expression of other proto-oncogenes in the respiratory epithelium. This knowledge could prove useful in formulating strategies to reduce the incidence of DNA damage in pollution-exposed populations, especially children and outdoor workers. This will not prove trivial since millions of people are exposed to low ozone levels for several hours a day, even though the current hourly ozone standard may not be exceeded.

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